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# US ARMY MEDICAL RESEARCH LABORATORY

FORT KNOX, KENTUCKY

REPORT NO. 572

## EFFECTS OF FERRIC NUCLEOTIDES ON MITOCHONDRIAL RESPIRATION

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UNITED STATES ARMY
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The animals used in this study were handled in accordance with the "Principles of Laboratory Animal Care" established by the National Society for Medical Research.

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# EFFECTS OF FERRIC NUCLEOTIDES ON MITOCHONDRIAL RESPIRATION

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Basic Research in Life Sciences
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## ABSTRACT

### EFFECTS OF FERRIC NUCLEOTIDES ON MITOCHONDRIAL RESPIRATION


#### OBJECT

The investigation of the effects of Fe(III)ATP, Fe(III)ADP, $Fe(III)_2ADP$, and $Fe(III)_3ADP$ on mitochondrial respiration.

#### RESULTS AND CONCLUSIONS

Addition of ferric adenosine diphosphates to liver mitochondria gave ADP:O values higher than those obtained with either Mg(II)ADP or ADP. Fe(III)ADP stimulated succinate oxidation to a level not obtained with Mg(II)ADP. Fe(III)ATP enhanced succinate oxidation when mitochondria were treated with AMP. The results of these studies indicate that ferric adenosine diphosphates stimulate respiration by providing a phosphate acceptor for tightly coupled mitochondria.

#### RECOMMENDATIONS

A continuation of investigations in the biochemical and physiological effects of ferric nucleotides.


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# EFFECTS OF FERRIC NUCLEOTIDES ON MITOCHONDRIAL RESPIRATION

## I. INTRODUCTION

Impressive quantities of non-heme iron have been found in mitochondria (1), submitochondrial respiratory complexes (2), and soluble succinic dehydrogenase (3). Part of this non-heme iron has been reported to be reduced by either succinate or NADH* (4). Other evidence supporting the participation of non-heme iron in electron transport was reviewed by Green (5). It remains to be determined whether non-heme iron functions in oxidative phosphorylation or electron transport of tightly coupled mitochondria.

Investigations of the interaction between respiratory enzymes and exogenous iron have been hindered by the low solubility of ferric hydroxide, about $10^{-15}$ M at pH 7, and by the rapid oxidation of ferrous iron in the presence of oxygen. The existence of a variety of ferric nucleotides (6, 7, 8), which are soluble at physiological hydrogen ion concentrations, suggested a new approach for the study of non-heme iron in oxidative phosphorylation. The present communication describes the effects of Fe(III)ATP, $Fe(III)_3ADP$, $Fe(III)_2ADP$, and Fe(III)ADP on mitochondrial respiration. These studies revealed that ferric adenosine diphosphates give higher ADP:O values and stimulate succinate oxidation to a higher level than that observed with Mg(II)ADP.

## II. MATERIALS AND METHODS

Respiratory measurements. Rat liver mitochondria were isolated in 0.25 M sucrose by the method of Schneider (9). Respiration was measured polarographically in a sealed, glass vessel surrounded by a water jacket at 25°C (10). ADP:O values were determined by the procedure of Chance and Williams (11). In comparing the effects of two reagents, the experiments were performed alternately on mitochondria from the same rat to minimize variability. The ADP:O values for α-ketoglutarate oxidation were not measured until at least 15 minutes after isolation of mitochondria so that Mg(II)ADP:O values would be low.

All reaction media contained 50 mM sucrose, 40 mM KCl, and 20 mM potassium phosphate, pH 7.4. The Mg(II) medium had 10 mM $MgCl_2$. When α-ketoglutarate was substrate, 10 mM sodium malonate was included. In measurements of substrate-ADP respiration, ADP

*Abbreviations are given in paragraph VII, page 11.

(> 250 μM) and respiratory substrate (15 mM) were present in sufficient excess to permit maximal respiration.

Reagents. Ferric nucleotides were formed in either an acidic or alkaline medium as described elsewhere (7, 8). Fe(III)ATP, Fe(III)-oxalacetate (12), Fe(III)EDTA, and some samples of Fe(III)ADP were formed in acid and neutralized. Fe(III)ADP, $Fe(III)_2$ADP, and $Fe(III)_3$-ADP were prepared by the alkaline method and neutralized. Fe(III)ADP made by either method gave the same results. $FeCl_3$ was used as the Fe(III) reagent. Concentrations of ferric nucleotides were determined spectrophotometrically and also calculated from the dilution of measured amounts of Fe(III) and nucleotides added to a given volume.

Spectrophotometric assays of ferric nucleotides were made in 10 mM $NH_4OH$-$NH_4Cl$ buffer, pH 9.4. At 259 mμ, Fe(III)ADP had an extinction coefficient of 19.4 $mM^{-1}cm^{-1}$ (8); values for other ferric adenosine disphosphates are reported elsewhere (8). The nucleotide concentration of stock solutions of ADP was determined using an extinction coefficient of 15.4 $mM^{-1}cm^{-1}$ for 259 mμ at pH 7 or 14.9 $mM^{-1}cm^{-1}$ for 257 mμ at pH 2.

The same batch of ADP was used to prepare the metal-ADP and ADP solutions used for ADP:O measurements. A possible AMP contamination would thus cause the same error in all measurements. Solutions were prepared in large amounts and kept frozen at -20°C until used (13). Phosphate determinations by the method of Fiske and Subbarow (14) revealed that these metal-ADP and ADP solutions had been dephosphorylated to the same degree (less than 2%).

Sodium ADP (98%), disodium ATP (99%), and cytochrome c, type II, were obtained from Sigma Chemical Company; L-cysteine sulfinic acid and sodium succinate, Grade A, from California Corporation for Biochemical Research; KCl, $KH_2PO_4$, $K_2HPO_4$, $MgCl_2$, and sucrose were Fisher Certified reagents from Fisher Scientific Company. The preparation and sources of other reagents were the same as indicated previously (15). Reagents were dissolved in deionized distilled water.

## III. RESULTS

Respiratory control by ferric adenosine disphosphates. Respiration of mitochondria in a Mg(II)-free medium was controlled by ferric adenosine diphosphates in a manner similar to that found by Baltscheffsky (16) for ADP. Figure 1a illustrates the burst of oxygen consumption caused

by the addition of Fe(III)ADP to mitochondria pretreated with β-hydroxybutyrate. A subsequent addition of Fe(III)ADP produced another burst. These findings show that the phase of rapid respiration terminated when the mitochondria became depleted of phosphate acceptor. The similarity of response to Fe(III)ADP, Mg(II)ADP (Fig. 1b), and ADP (1c) suggests that Fe(III)ADP was converted to ATP or its metal chelate.

The addition of Fe(III)ADP stimulated β-hydroxybutyrate oxidation to the same level (2.7 μmoles $O_2$/sec/g. N) as the addition of either Mg(II)ADP or ADP. The respiratory rate then decreased to a value which was somewhat greater than that observed before the addition of Fe(III)-ADP, Mg(II)ADP, or ADP. The increased respiratory rate after the burst appears to have resulted from incipient mitochondrial damage accompanying incubation in the Mg(II)-free medium (16).

In contrast to the system with β-hydroxybutyrate, Fe(III)ADP stimulated succinate respiration to a higher level than either ADP or Mg(II)ADP. Fe(III)ADP increased succinate respiration from 1 μmole $O_2$/sec/g. N to 11 μmoles $O_2$/sec/g. N, whereas ADP or Mg(II)ADP increased oxidation only to 7 μmoles $O_2$/sec/g. N. After the initial burst of oxygen uptake caused by adding Fe(III)ADP (Fig. 2a), the respiratory rate did not diminish to the value obtained in the system with either ADP (Fig. 2b) or Mg(II)ADP. $Fe(III)_2ADP$ and $Fe(III)_3ADP$ had the same effect on mitochondrial respiration as Fe(III)ADP.

Mg(II) in the reaction medium did not affect the stimulation of succinate oxidation by Fe(III)ADP. Consequently, additional measurements were performed in a medium containing 10 mM $MgCl_2$. In this medium the mitochondria remained tightly coupled for a longer time than in the Mg(II)-free medium. These studies revealed that the order of adding the nucleotides affected their action. Once a burst of respiration had been induced by Fe(III)ADP, then either Mg(II)ADP or Fe-(III)ADP produced the same high rate of respiration, i.e., 11 μmoles $O_2$/sec/g. N.

Only succinate respiration was affected in the manner described above. Oxidation of β-hydroxybutyrate, malate plus pyruvate, β-hydroxybutyrate plus malate plus pyruvate, α-ketoglutarate with a malonate block, or proline with a malonate block was stimulated equally effectively by either Mg(II)ADP or Fe(III)ADP. When mitochondria were treated with cytochrome c, ADP (500 μM), and substrate, the addition of Fe(III)ADP (500 μM) caused no further stimulation except when succinate was oxidized.

These findings suggest that Fe(III)ADP has at least two effects on mitochondria. First Fe(III)ADP stimulates respiration of all substrates by providing a phosphate acceptor. In addition, Fe(III)ADP stimulates succinate oxidation to a higher level than ADP.

ADP:O values. The characteristic respiratory response of mitochondria to phosphate acceptor permitted comparing Fe(III)ADP:O, Mg(II)ADP:O, and ADP:O values. In the oxidation of succinate, Fe(III)-ADP:O values were measured in the manner shown in Figure 2a. Only the second and third bursts were used to calculate succinate Fe(III)-ADP:O values, because of the increase in respiration after the first burst of oxidation.

The data presented in Table 1 show that both Fe(III)ADP:O and $Fe(III)_3$ADP:O values were 20% higher than either Mg(II)ADP:O or ADP:O values for α-ketoglutarate, β-hydroxybutyrate, and succinate oxidation. Alternating the order of addition of nucleotides did not change these results. When Mg(II)ADP was added after a respiratory burst caused by a ferric adenosine diphosphate, the Mg(II)ADP:O value remained lower than the Fe(III)ADP:O value.

The conditions used for these measurements were selected so that the Mg(II)ADP:O and ADP:O values were relatively low. For example, β-hydroxybutyrate and succinate ADP:O values were measured in a Mg(II)-free medium. Under these conditions the ADP:O and Mg(II)ADP:O values, but not Fe(III)ADP:O values, were lower than in a medium containing Mg(II).

For measurements in the Mg(II)-free medium, the respiration after the burst caused by adding nucleotide was slightly greater than the initial respiration, especially for β-hydroxybutyrate oxidation. These observations indicate that the phosphate acceptor level was slightly higher after the burst than before (11, 15). This does not prevent comparing Fe(III)ADP:O values with ADP:O values, because the error introduced was the same. Also the affinity of mitochondria for ADP is sufficiently high (11) that the error is small.

The higher ADP:O values obtained with ferric adenosine diphosphates were not given by a number of other metal-ADP compounds. Co(II)ADP:O, Ni(II)ADP:O*, and Mn(II)ADP:O values were the same as, or lower than, Mg(II)ADP:O ratios.

*Unpublished experiments indicate the existence of a Ni(II)ADP complex.

Adenylate kinase activity. To aid in interpreting the significance of the high Fe(III)ADP:O values, some additional reactions of ferric nucleotides were studied. Since Fe(III)ADP was shown to stimulate mitochondrial respiration, oxygen consumption could be used to determine adenylate kinase activity. Figure 3 shows that adding either AMP or Fe(III)ATP caused only slight initial stimulation of succinate oxidation. When both AMP and Fe(III)ATP were added, respiration immediately increased more than twofold. These findings suggest that the presence of Fe(III) does not prevent adenylate kinase activity.

Adenylate kinase activity was used to test for the formation of either Fe(III)ATP or ATP during the burst of respiration caused by Fe(III)ADP. Mitochondria were treated with succinate and Fe(III)ADP (110 μM) in a Mg(II)-free medium. After the ensuing respiratory burst, the addition of AMP (160 μM) immediately caused a second respiratory burst. Thus ATP was formed during the burst of oxidation caused by adding Fe(III)ADP. This Fe(III)ATP, or ATP, probably was not formed by adenylate kinase action on 2 Fe(III)ADP, because the resulting AMP would have prevented termination of the first burst.

Effects of dinitrophenol and inhibitors on Fe(III)-stimulated succinate oxidation. Studies were undertaken to attempt to clarify the mechanism involved in Fe(III) stimulation of succinate oxidation. The combined effects of Fe(III)ADP and the uncoupling agent dinitrophenol are summarized in Table 2. Dinitrophenol stimulated succinate oxidation to the same level as Fe(III)ADP. Addition of Fe(III)ADP, but not ADP, to mitochondria treated with dinitrophenol increased succinate oxidation by 20%. Reversing the order of adding the reagents did not alter the final result. Addition of dinitrophenol caused a slight stimulation of succinate-Fe(III)ADP (520 μM) respiration.

The data of Table 2 further show that cysteine sulfinate (17) and amytal, which prevented oxalacetate inhibition in uncoupled mitochondria, did not block the stimulation of succinate-ADP respiration by Fe(III)ADP. Treating mitochondria with cysteine sulfinate (4.7 mM) for 3 minutes before adding amytal (1-5 mM) and succinate did not prevent this Fe(III)-ADP effect.

Phenethylbiguanide (1 mM), which affects only respiration coupled to phosphorylation (18, 19), inhibited succinate-Fe(III)ADP respiration. This inhibition was overcome by dinitrophenol (60 μM) in the presence of cysteine sulfinate (4.7 mM). Alternatively the inhibition was greatly reduced by increasing the concentration of Fe(III)ADP (Table 3). When the phenethylbiguanide concentration was low, the inhibition of succinate-ADP

respiration was completely reversed by Fe(III)ADP, but not by ADP (Fig. 4). The presence of both phenethylbiguanide and Fe(III)ADP did not appear to cause uncoupling, since Fe(III)ADP still produced respiratory bursts. These findings suggest that Fe(III)ADP may displace phenethylbiguanide from its site of inhibition.

The stimulation of succinate respiration by Fe(III)ADP was prevented by 10 mM malonate, an inhibitor of succinic dehydrogenase. Respiratory inhibition by lower concentrations of malonate is shown in Figure 5. A concentration of malonate which reduced succinate-Fe(III)-ADP respiration to the level of succinate-ADP respiration caused less than 10% decrease in succinate-Fe(III)ATP respiration. In contrast, the initial addition of Fe(III)ADP led to a 30% higher rate of succinate respiration after the burst. Consequently, Fe(III) may also affect the ATPase activity which controls succinate respiration in the absence of added phosphate acceptor (15, 20). This does not mean that ATPase activity in the presence of Fe(III)ADP is increased, because ATPase activity may be greatly reduced during rapid oxidative phosphorylation (11).

Effects of metal chelates on succinate oxidation. Fe(III)ATP, Fe(III)oxalacetate, or Fe(III)EDTA did not cause a burst of mitochondrial respiration. These Fe(III) compounds, however, did enhance succinate-ADP respiration to the same extent as Fe(III)ADP (Fig. 6). Half maximal effects were obtained at 40 μM Fe(III)ADP, Fe(III)ATP, or Fe(III)oxalacetate. ATP or oxalacetate at these concentrations had no effect. Addition of Fe(III)EDTA at much higher concentrations also stimulated respiration. This effect may have been caused by EDTA (see below) or by a displacement of Fe(III) from Fe(III)EDTA to ADP (8). When respiration was maximally stimulated by Fe(III)ADP, addition of the other chelates caused no further stimulation.

Numerous metal compounds enhanced succinate-ADP respiration (Fig. 7). Al(III)ADP*, Ba(II)ADP, or Ca(II) (21) raised succinate oxidation to the same rate as Fe(III)ADP. Additions of either Co(II)ADP (22) or Mn(II)ADP (22) increased succinate oxidation to a lesser extent. Ni(II)ADP (270 μM), which stimulated succinate respiration to the same extent as Mg(II)ADP, did not alter succinate-ADP respiration.

Al(III)ADP (250 μM) did not affect succinate-Fe(III)ADP (250 μM) respiration. Addition of Fe(III)ADP (250 μM) did not stimulate succinate-Al(III)ADP (250 μM) respiration. Co(II)ADP (610 μM) inhibited succinate-

*Investigations were not made to establish the existence of an Al(III)ADP complex.

Fe(III)ADP (590 μM) respiration by 35%. Conversely, Fe(III)ADP (590 μM) enhanced succinate-Co(II)ADP (305 μM) respiration by 30%. Addition of Ni(II)ADP to mitochondria pretreated with succinate and Fe(III)ADP decreased oxygen consumption to the value obtained with Mg(II)ADP (Fig. 8).

Serial additions of Co(II)ADP, Al(III)ADP, Mn(II)ADP, Ba(II)ADP, or Ni(II)ADP produced a series of respiratory bursts. Oxygen consumption was the same before and after the burst of respiration caused by Co(II)ADP. With Al(III)ADP, Ba(II)ADP, or Mn(II)ADP, respiration after the first burst was 30% greater than the initial rate.

Addition of Zn(II)ADP (23) or Cu(II)ADP (23) inhibited succinate oxidation. Marked inhibition occurred immediately after adding Zn(II)ADP (270 μM). Treating mitochondria with Cu(II)ADP enhanced respiration for 30 sec before inhibition occurred (Fig. 9).

Effects of EDTA on succinate oxidation. Attempts were made to increase the disparity between the action of Fe(III)ADP and Mg(II)ADP on succinate respiration. Mitochondria were isolated from livers homogenized in 250 mM sucrose containing 1 or 10 mM EDTA, pH 7. The preparations were washed twice in 250 mM sucrose to remove EDTA from the mitochondria. These treatments were without effect on succinate oxidation.

Yet addition of EDTA to the reaction medium increased succinate-ADP respiration, but had no effect on β-hydroxybutyrate-ADP, α-ketoglutarate-ADP, or pyruvate-malate-ADP respiration. With the 10 mM Mg(II) medium, as little as 0.015 to 1 mM EDTA raised succinate-ADP respiration to the same level as Fe(III)ADP. Half maximal stimulation was given by 5 μM EDTA. Succinate-ADP respiration in the Mg(II)-free medium was also enhanced by EDTA (30 μM). No further stimulation occurred when EDTA (1 mM) was added to mitochondria treated with succinate, Mg(II), and Fe(III)ADP (0.2 mM). Conversely, Fe(III)ADP (1 mM) did not alter succinate-ADP-EDTA (0.3 mM) respiration. EDTA (30 μM) caused a 20% increase in succinate oxidation by mitochondria treated with dinitrophenol (100 μM), cysteine sulfinate (4.7 mM), and amytal (1.0 mM).

## IV. DISCUSSION

The results of the present investigation indicate that ferric adenosine diphosphates stimulate respiration by providing a phosphate acceptor for tightly coupled mitochondria. Serial additions of Fe(III)ADP,

$Fe(III)_2ADP$, or $Fe(III)_3ADP$ caused corresponding bursts of oxygen uptake. This respiratory response is characteristic of compounds that cause concomitant stimulation of respiration and phosphorylation (11, 21, 24, 25). Studies utilizing the adenylate kinase activity of mitochondria confirmed that ATP or its metal chelate is formed from Fe(III)ADP during the respiratory bursts. The inhibition of succinate-Fe(III)ADP respiration by phenethylbiguanide provided additional evidence that phosphorylation is coupled with respiration.

The manner in which ferric adenosine diphosphates stimulate oxidative phosphorylation is uncertain. These nucleotides may participate directly in the process of oxidative phosphorylation. On the other hand, transphosphorylation reactions could occur between endogenous ATP and Fe(III)ADP to yield ADP. This reaction, however, appears unlikely because the similar adenylate kinase-catalyzed reaction between added AMP and endogenous ATP is initially slow. Another possibility is that metals bound to the mitochondria (1) may displace the Fe(III) from ferric adenosine diphosphates before the phosphorylation occurs.

In this connection, the observation that the addition of $Fe(III)_2ADP$ and $Fe(III)_3ADP$ caused respiratory bursts is intriguing. The presence of 2 or 3 iron atoms must greatly alter the charge density and steric configuration of ADP (8). Yet the addition of either $Fe(III)_2ADP$ or $Fe(III)_3ADP$ stimulated respiration to the same extent as the addition of Fe(III)ADP. If the polynuclear ferric adenosine diphosphates are phosphorylated, the lack of specificity for phosphate acceptor is surprising. When the base moiety of ADP is altered, marked differences in the rate of oxidative phosphorylation are observed (26).

Ferric adenosine diphosphates gave higher ADP:O values than did Mg(II)ADP. By analogy with the case for Mg(II)ADP (11, 27), Fe(III)-ADP:O values may be a quantitative measure of oxidative phosphorylation. Thus, Fe(III)ADP may be phosphorylated more efficiently than Mg(II)ADP. This might result from a difference in mitochondrial ATPase activity on the two metal-ATP compounds. When mitochondria are exposed to sub-optimal conditions, the normally occurring increase in ATPase activity during the respiratory burst may be depressed by Fe(III)ADP. Further studies are needed to determine the extent to which other factors, such as the possible reduction of Fe(III) (28), may influence Fe(III)ADP:O values.

Ferric adenosine diphosphates enhanced succinate respiration to a greater extent than Mg(II)ADP. A number of metal chelates and also

EDTA stimulated succinate oxidation in mitochondria treated with excess ADP. No combination of these compounds caused a higher rate of succinate oxidation than Fe(III)ADP alone.

The simplest hypothesis explaining these results is that the mitochondria contain an inhibitory ion, since all of the active agents are capable of displacing cations. Impurities in the reagents may be the source of the inhibitory ion, because EDTA had to be present in the reaction medium to be stimulatory. Fe(III), Al(III), Ba(II), or Ca(II) would displace the hypothetical ion. Also Co(II) and Mn(II) would be able to displace the cation, but are themselves slightly inhibitory. Apparently EDTA forms a highly stable chelate with the inhibitory ion. Ni(II) has many characteristics of the hypothetical ion, and its EDTA chelate has a stability constant $10^{10}$ times greater than that of Mg(II)-EDTA (29).

Neither Fe(III)ADP nor EDTA enhanced the rate of oxidation of NAD-linked substrates by mitochondria previously treated with ADP. These results may suggest that the inhibitory ion acts between succinate and a point where the succinate pathway joins the NAD-linked pathway (30). On the other hand, the oxidation of NAD-linked substrates is less rapid than the oxidation of succinate. Reagents that increase the rate of succinate oxidation might not cause a measurable increase in the rate of oxidation of NAD-linked substrates, even if some step common to the two systems is affected.

Dinitrophenol caused an increase in succinate oxidation to the level obtained with either Fe(III)ADP or ADP plus EDTA. Chappell (31), who first reported this type of dinitrophenol effect, suggested that the phosphorylative steps limit oxidation of succinate, but not the oxidation of most NAD-linked substrates. The results of the present investigation suggest that the effects of dinitrophenol may be more complex. In addition to the uncoupling action of dinitrophenol (32), it may also react with an inhibitory ion. Dinitrophenol has a structure which favors a stable chelate. Even phenol forms a complex with Fe-(III) (33). Much of the stimulation of succinate-ADP respiration by dinitrophenol may result from a possible chelating action. Uncoupling is not a necessary requirement for stimulation of succinate-ADP respiration, since tightly coupled mitochondria also show the effect. A reaction of dinitrophenol with a metal would account for the failure of either EDTA or Fe(III)ADP to cause appreciable stimulation of succinate oxidation in mitochondria treated with dinitrophenol. Thus the studies with dinitrophenol, Fe(III)ADP, and EDTA provide no evidence that the inhibitory ion acts at the phosphorylative steps.

The observation that EDTA stimulated succinate oxidation has been reported for non-phosphorylating heart preparations (34, 35). Also an effect of Al(III) and Ca(II) on the non-phosphorylating succinoxidase system is well known (36). The stimulation by this combination of ions has been attributed to their preventing oxalacetate inhibition (36). In the present study, the stimulation of succinate oxidation by Fe(III) in tightly coupled mitochondria apparently did not result from removal of oxalacetate inhibition. Cysteine sulfinate and amytal, which prevent oxalacetate inhibition, did not decrease in the Fe(III) effect.

Another effect of Fe(III)ADP may be of value in locating the site of phenethylbiguanide action on oxidative phosphorylation. The inhibition of respiration by phenethylbiguanide was relieved by Fe(III)-ADP. The degree of this respiratory inhibition may depend on a competition between Fe(III)ADP and phenethylbiguanide. These findings suggest that Fe(III)ADP may be able to displace phenethylbiguanide from its site of action. This displacement might result either from an interaction between Fe(III)ADP and the site of phenethylbiguanide inhibition or from complex formation of Fe(III)ADP with phenethylbiguanide.

## V. SUMMARY

Addition of ferric adenosine diphosphates to rat liver mitochondria induced a burst of respiration similar to that given by ADP. The ratio of moles of added Fe(III)ADP to gram-atoms of oxygen removed during the burst was 20% greater than in the corresponding case with ADP. Fe(III)ADP produced the same respiratory stimulation as Mg(II)ADP with malate plus pyruvate, β-hydroxybutyrate, α-ketoglutarate, or proline as substrate. Fe(III)ADP enhanced succinate respiration 60% more than Mg(II)ADP. When mitochondria were treated with succinate and excess ADP, either Fe(III)ATP or Fe-(III)oxalacetate stimulated respiration as much as Fe(III)ADP. Several other metal-ADP compounds and EDTA plus ADP also increased succinate oxidation more than Mg(II)ADP. Respiration of mitochondria treated with Fe(III)ADP was inhibited by high concentrations of phenethylbiguanide. Fe(III)ADP reversed the respiratory inhibition caused by low concentrations of phenethylbiguanide.

## VI. ACKNOWLEDGEMENT

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## VII. ABBREVIATIONS

μM, micromole per liter; mM, millimole per liter; AMP, ADP, ATP, adenosine-5'-mono-, di-, and triphosphate; NAD, NADH, nicotinamide adenine dinucleotide and reduced form; EDTA, disodium ethylenediaminetetraacetate; Fe(III)ADP, $Fe(III)_2ADP$, $Fe(III)_3ADP$, mono-, di-, and triferric ADP; Fe(III)ADP:O, ADP:O, Mg(II)ADP:O, etc., moles of added nucleotide per gram-atom of oxygen consumed during the respiratory burst; ATPase activity, all reactions dephosphorylating ATP to either ADP or AMP. The state of mitochondrial respiration is indicated by listing the reagents added to the system. For example, succinate-ADP respiration represents respiration of mitochondria treated with succinate and ADP. Concentrations of reagents are given in parentheses where such information is pertinent.

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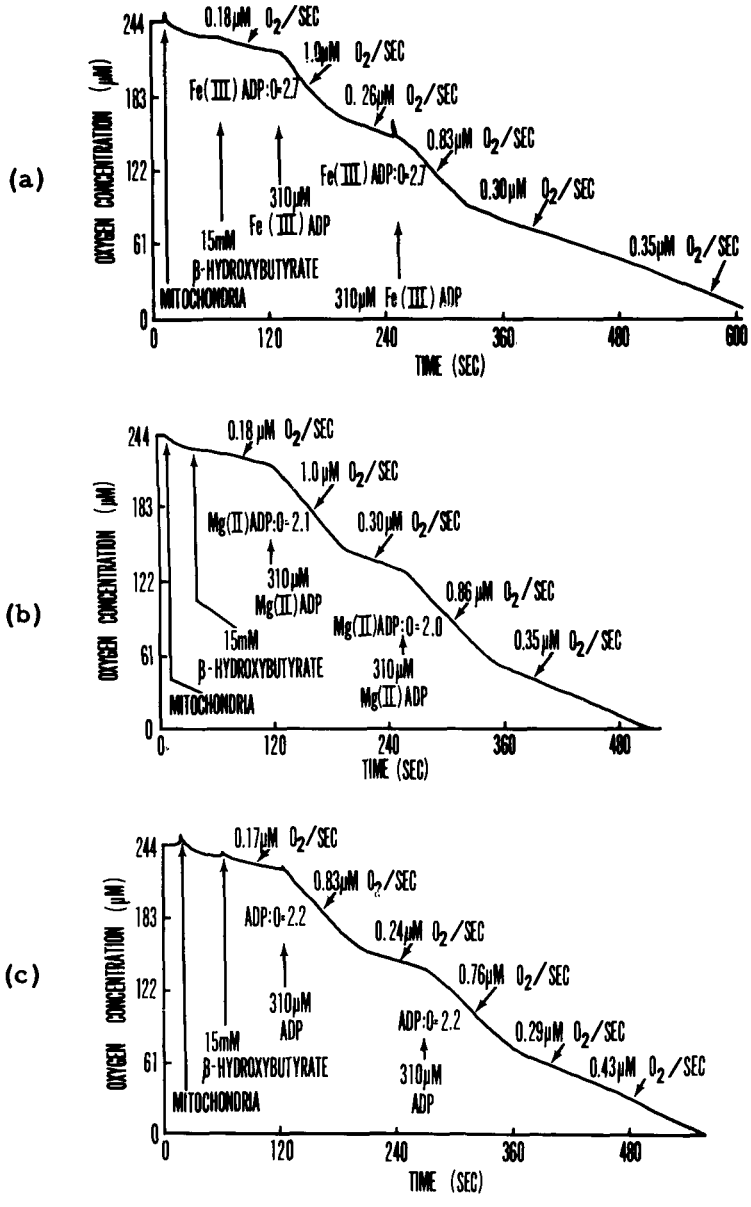


Fig. 1. Comparison of effect of Fe(III)ADP (a), Mg(II)ADP (b), and ADP (c) on β-hydroxybutyrate respiration. All reaction media contained 50 mM sucrose, 40 mM KCl, 20 mM potassium phosphate, pH 7.4. In Figure 1 a and b, 7.5 mg mitochondrial protein was added to 3.5 ml of medium; in Figure 1 c, 7.1 mg protein was added.

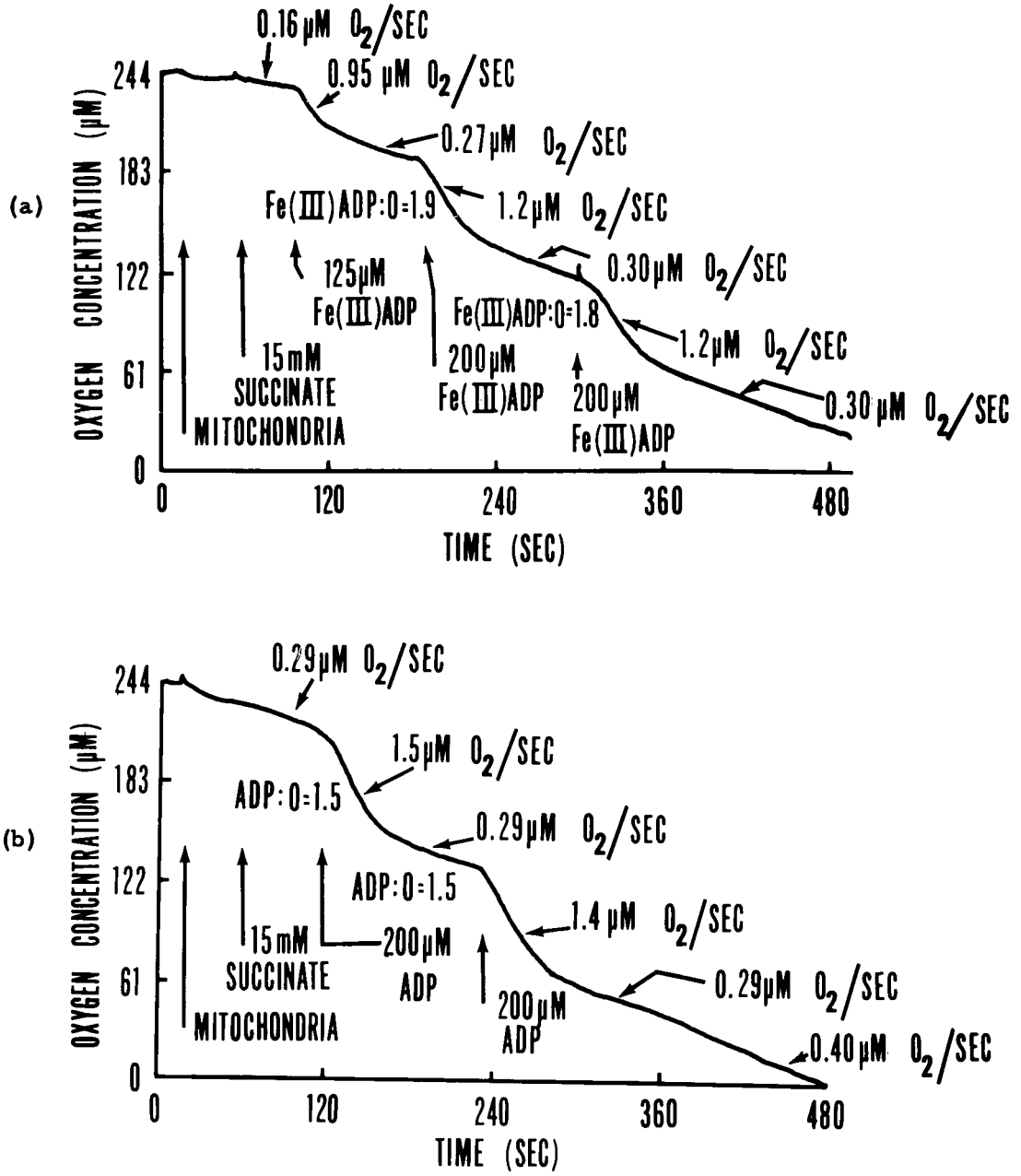


Fig. 2. Comparison of effect of Fe(III)ADP (a) and ADP (b) on succinate respiration. Both reaction media were the same as described in Figure 1. In Figure 2 a, 2.9 mg protein per 3.5 ml; in Figure 2 b, 6.2 mg protein per 3.5 ml.

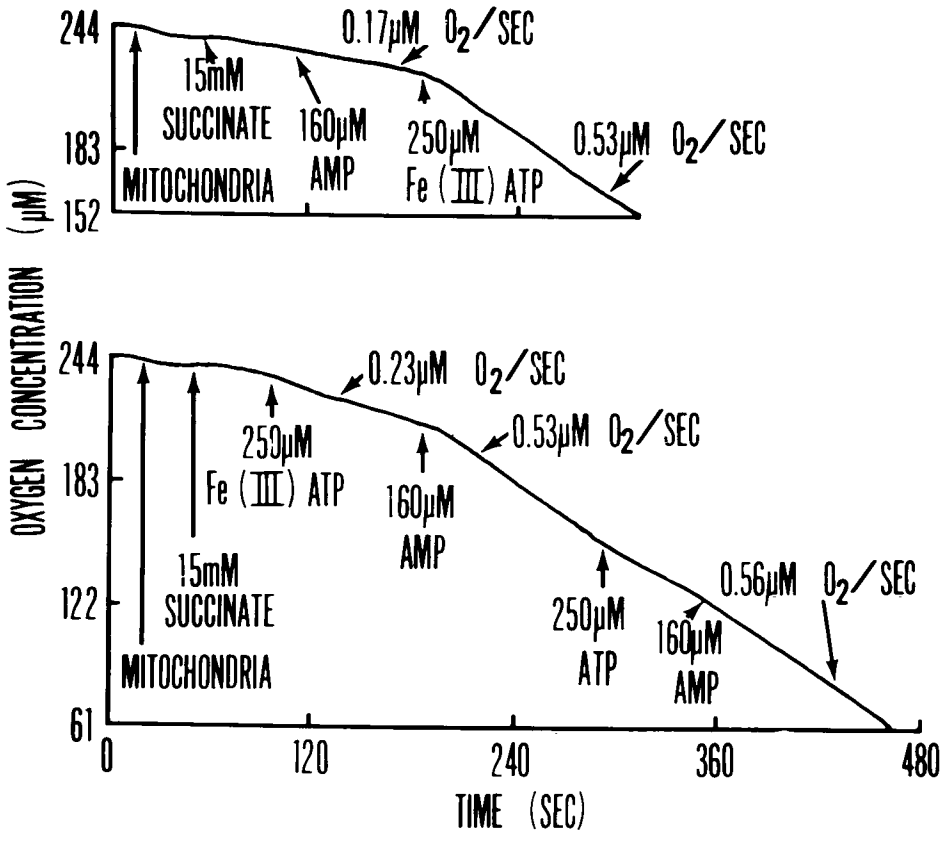


Fig. 3. Stimulation of succinate oxidation by Fe(III)ATP and AMP. The reaction medium contained 30 μM cytochrome c, 50 mM sucrose, 40 mM KCl, 20 mM potassium phosphate, pH 7.4. Approximately 1.5 mg mitochondrial protein was added to 3.5 ml of medium. If Fe(III)ATP had not been added to the medium in the top part of this figure, respiration would have increased gradually because of the low concentration of endogenous ATP.

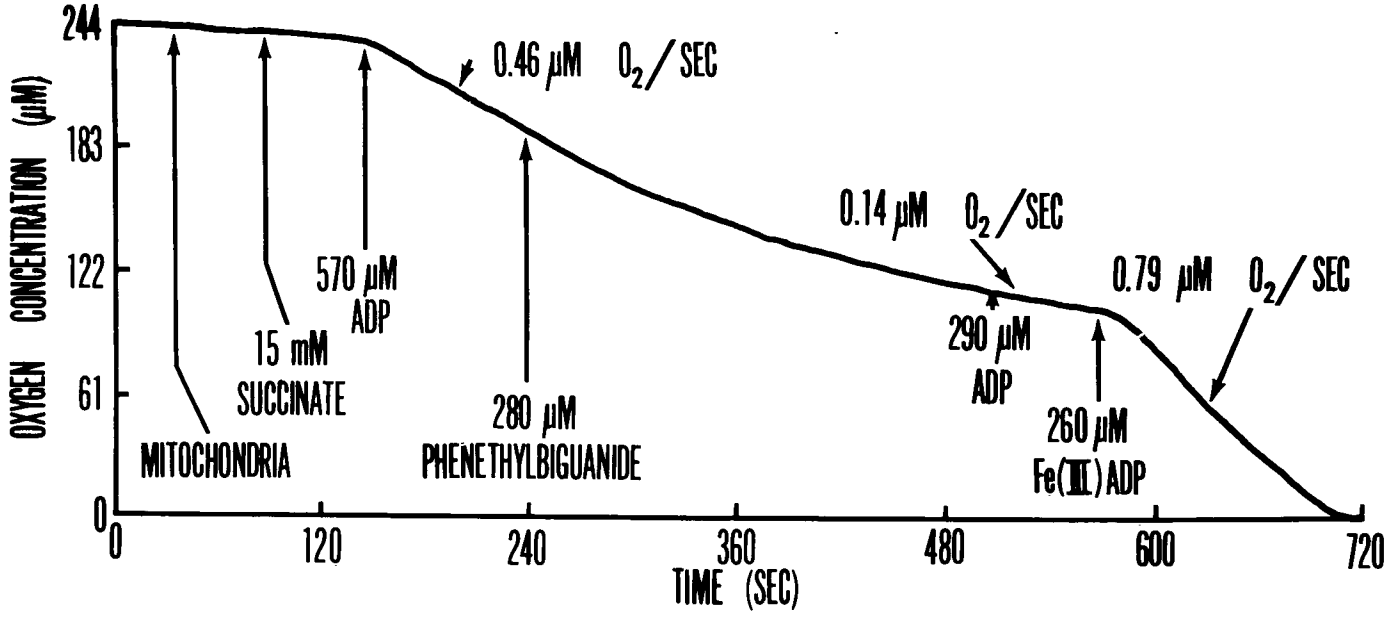


Fig. 4. Effect of Fe(III)ADP on succinate oxidation at a low concentration of phenethylbiguanide. The reaction medium contained 50 mM sucrose, 40 mM KCl, 20 mM potassium phosphate, 10 mM $MgCl_2$ and 30 μM cytochrome c, pH 7.4.

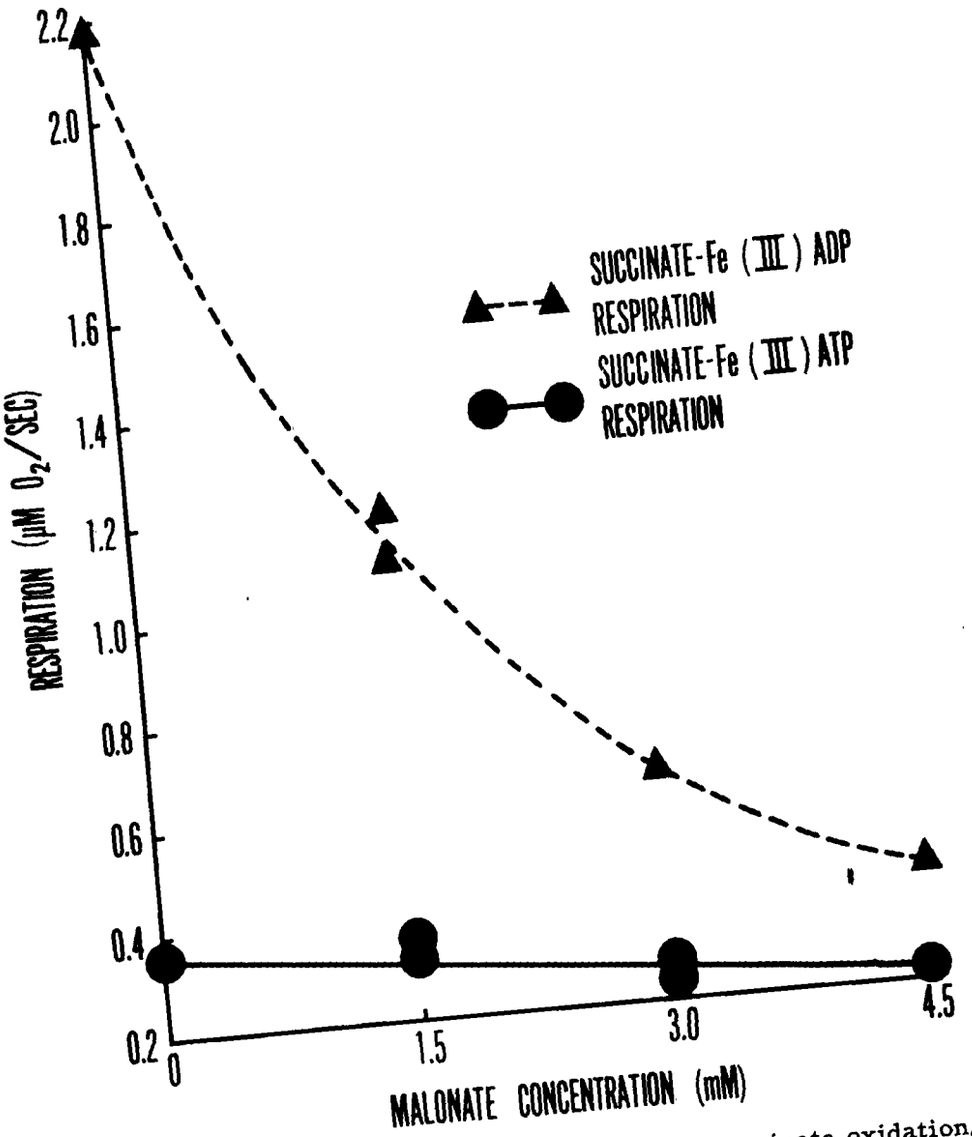


Fig. 5. Malonate inhibition of Fe(III)-stimulated succinate oxidation. The reaction medium contained 15 mM succinate, 50 mM sucrose, 40 mM KCl, 10 mM $MgCl_2$, 20 mM potassium phosphate, 4.5 mg protein per 3.5 ml, pH 7.4. Addition of Fe(III)ADP (250 μM) gave succinate-Fe(III)ADP respiration. Succinate-Fe(III)ATP respiration refers to respiration after the burst caused by Fe(III)ADP.

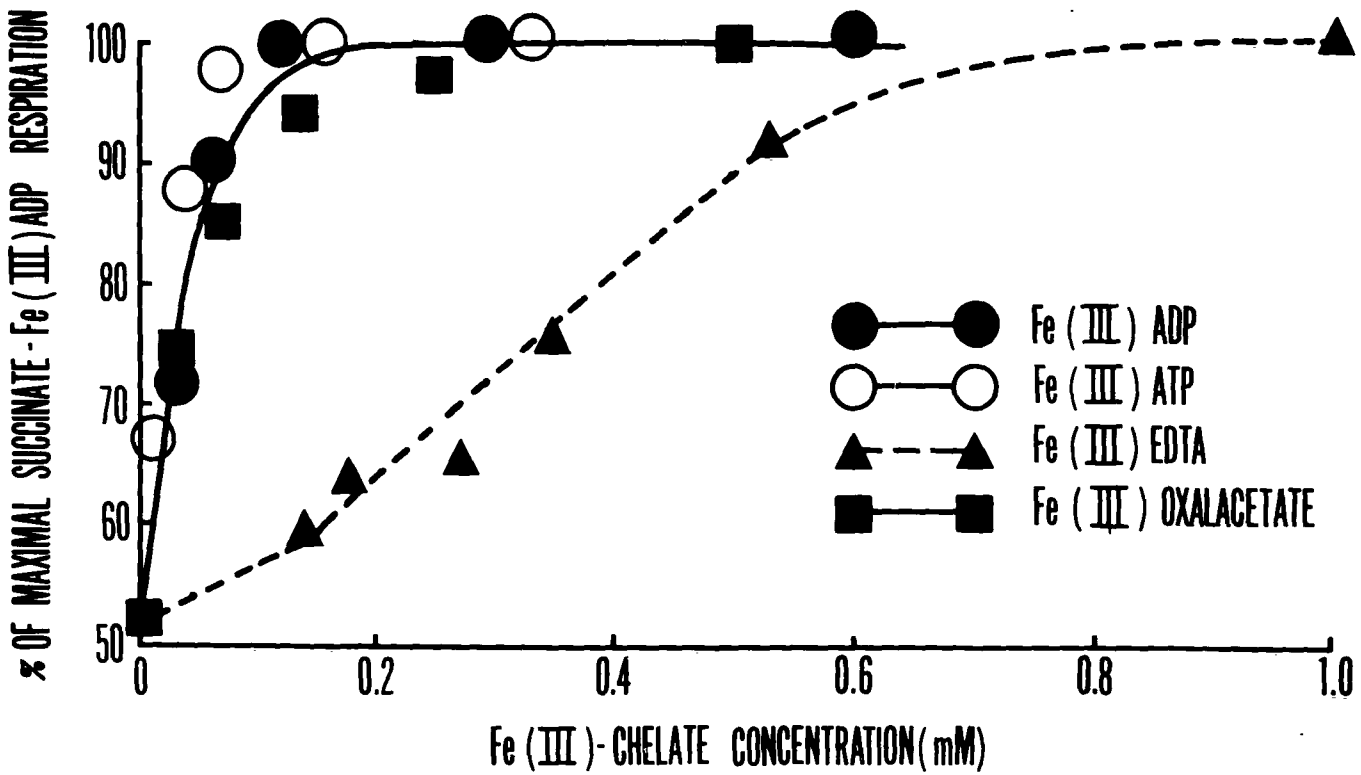


Fig. 6. Stimulation of succinate-ADP respiration by Fe(III) chelates. The reaction medium contained 15 mM succinate, 0.5 mM ADP, 30 μM cytochrome c, 50 mM sucrose, 40 mM KCl, 10 mM $MgCl_2$, 20 mM potassium phosphate, pH 7.4.

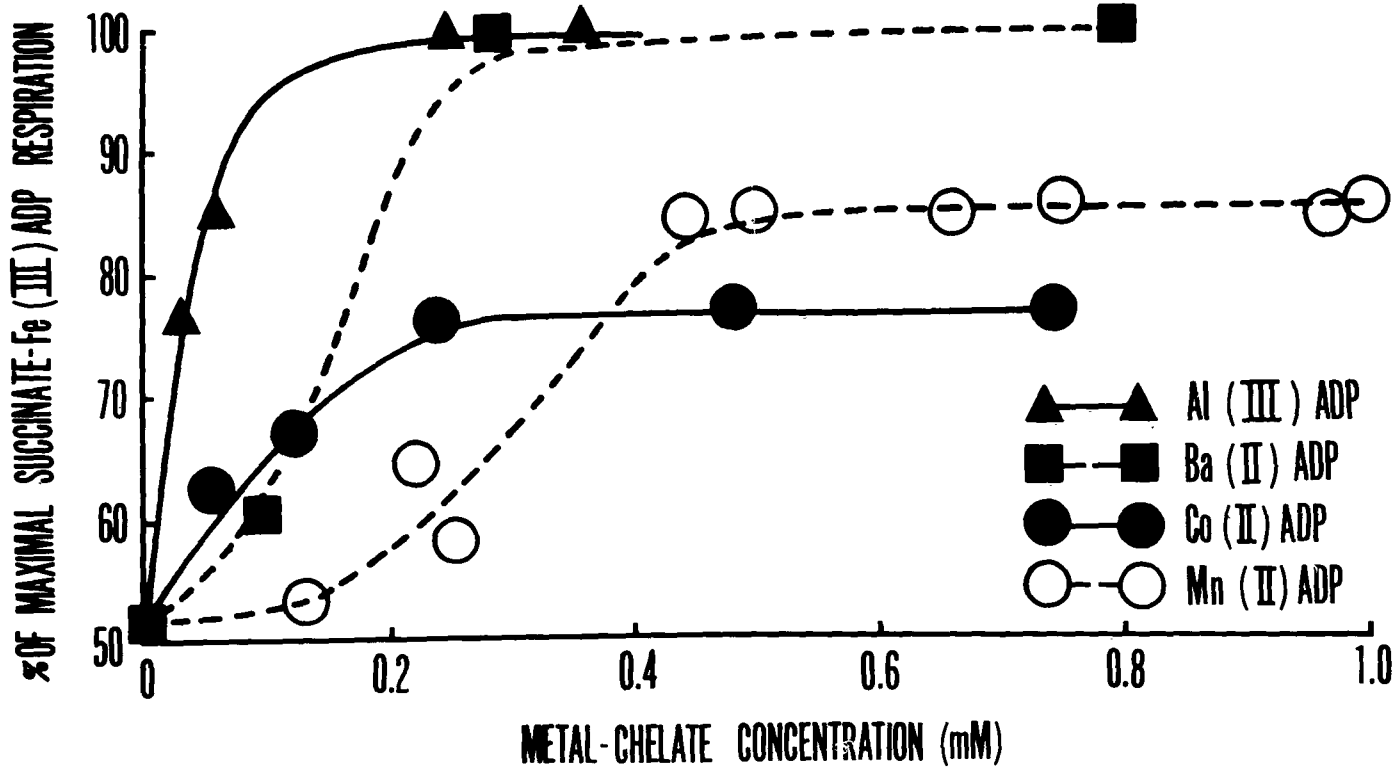


Fig. 7. Stimulation of succinate-ADP respiration by metal-ADP compounds. Conditions were the same as those given in legend of Figure 6.

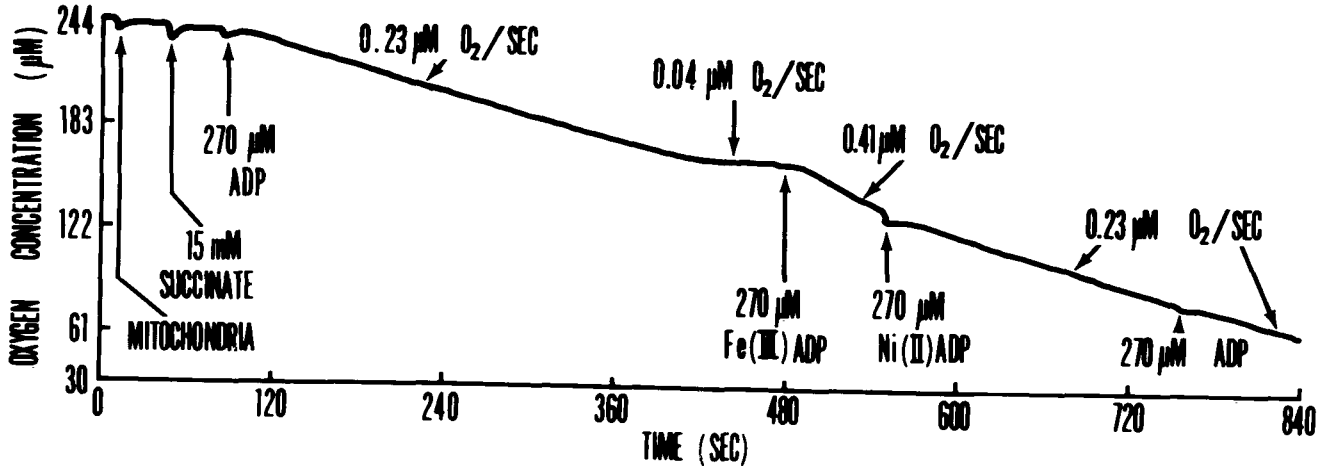


Fig. 8. Inhibition of succinate-Fe(III)ADP respiration by Ni(II)ADP. The reaction medium contained 50 mM sucrose, 40 mM KCl, 10 mM $MgCl_2$, 20 mM potassium phosphate, pH 7.4.

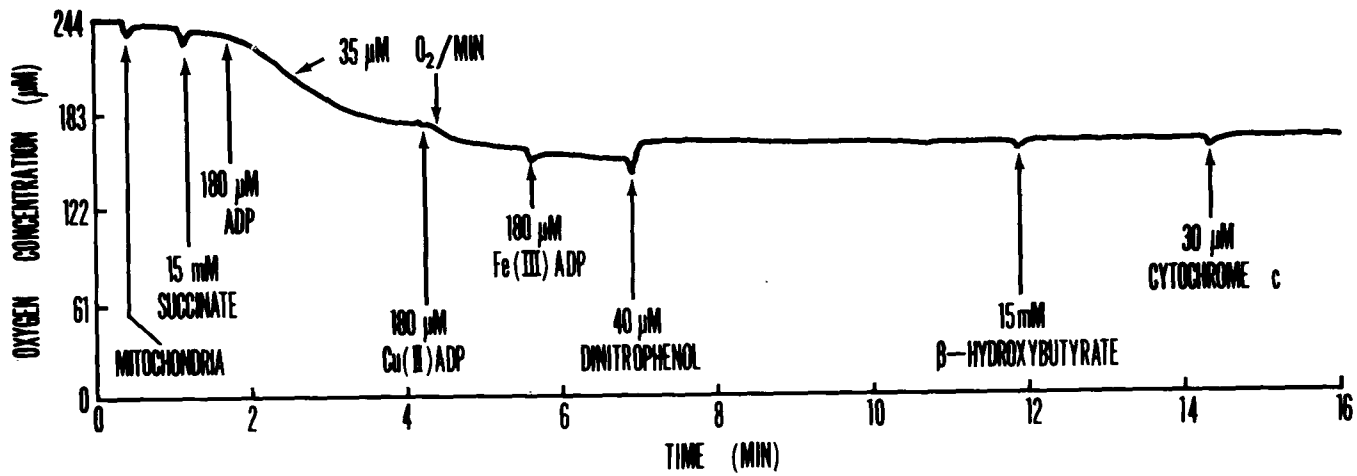


Fig. 9. Effect of adding Cu(II)ADP to mitochondria. The medium was the same as that given in the legend of Figure 8.

## TABLE 1

### ADP:O VALUES

Values for succinate and β-hydroxybutyrate were measured in the Mg(II)-free medium; for α-ketoglutarate, a Mg(II) medium with 10 mM malonate was used. Usually only the ADP:O values for the first additions of nucleotide were included in the data for β-hydroxybutyrate and succinate, because Mg(II)ADP:O and ADP:O decreased to a greater extent than did Fe(III)ADP:O for the second additions. Increase in respiration after the burst compared with respiration before the burst was: for succinate with $Fe(III)_3ADP$, Fe(III)ADP, or ADP, 5%, with Mg(II)-ADP, 20%; for β-hydroxybutyrate with Fe(III)ADP, Mg(II)ADP, or ADP, 45%, with $Fe(III)_3ADP$, 20%; for α-ketoglutarate with any nucleotide, 0%. Data were obtained on nine different mitochondrial preparations (9 rats). In all cases, the Fe(III)ADP:O values were higher than Mg(II)ADP:O and ADP: O values.

| Substrate | Fe(III)ADP:O* | $Fe(III)_3ADP$:O* | Mg(II)ADP:O* | ADP:O* |
|---|---|---|---|---|
| Succinate | 1.8±0.1(11) | 2.1±0.1(3) | 1.4±0.1(5) | 1.4±0.1(7) |
| β-hydroxy-butyrate | 2.8±0.1(6) | 3.1±0.2(3) | 2.3±0.1(5) | 2.3±0.1(3) |
| α-keto-glutarate | 3.6±0.2(10) | 3.7±0.2(5) | 3.0±0.1(14) | |

*Mean ± standard deviation. Number of measurements is shown in parentheses.

TABLE 2

EFFECT OF DINITROPHENOL AND Fe(III)ADP ON SUCCINATE OXIDATION

Mitochondria were added to a medium which contained 15 mM succinate, 30 μM cytochrome c, 1.0 mM amytal, 4.7 mM cysteine sulfinate, 50 mM sucrose, 40 mM KCl, 20 mM potassium phosphate, 10 mM $MgCl_2$, and, where indicated, 0.1 mM dinitrophenol, 0.52 mM Fe(III)ADP, 0.58 mM ADP, at pH 8.0.

| | RESPIRATION* | | |
|---|---|---|---|
| Succinate-ADP | Succinate-Fe(III)ADP | Succinate-dinitrophenol | Succinate-dinitrophenol-Fe(III)ADP |
| | μmoles $O_2$/sec/g. N | | |
| 6.8(2) | 11.0±0.7(3) | 10.4±0.5(5) | 12.6±0.2(4) |

*Mean ± standard deviation. Number of measurements is given in parentheses.

TABLE 3

REVERSAL OF PHENETHYLBIGUANIDE INHIBITION BY Fe(III)ADP

The reaction medium contained 1.0 mM phenethylbiguanide, 15 mM succinate, 0.6 mM ADP, 1.0 mM amytal, 4.7 mM cysteine sulfinate, 30 μM cytochrome c, 50 mM sucrose, 40 mM KCl, 20 mM potassium phosphate, 10 mM $MgCl_2$, 1.2 mg mitochondrial protein/3.5 ml, at pH 7.4. Measurements were made after inhibition reached an approximately constant level.

| Fe(III)ADP Concentration, mM | % of Succinate-Fe(III)ADP Respiration |
|---|---|
| 0 | 5 |
| 0.26 | 30 |
| 0.52 | 40 |
| 1.0 | 55 |

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US Army Medical Research Lab, Ft. Knox, Ky.
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Report No. 572, 20 May 63, 25 pp & i - 9 illus - 3 tables - DA Project No. 3A013001A814, Unclassified Report

UNCLASSIFIED
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2. Electron transport
3. Ferric nucleotides

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